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(54) Title: SEQUENCE-SPECIFIC NONPHOTOACTIVATED CROSSLINKING AGENTS WHICH BIND TO THE MAJOR GROOVE OF DUPLEX DNA

(57) Abstract

Agents which bind to the major groove of nucleic acid duplexes in a sequence-specific manner and are capable of forming covalent bonds with one or both strands of the duplex in the absence of light are useful therapeutic agents in the treatment of conditions mediated by duplex DNA. These agents are designed so that the reactivity of the crosslinking agent does not interfere with the sequence specificity of the agent which binds to the major groove. Thus, specific desired DNA duplexes can be targeted and their activity diminished or enhanced.

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SEQUENCE-SPECIFIC NONPHOTOACTIVATED CROSSLINKING AGENTS WHICH BIND TO THE MAJOR GROOVE OF DUPLEX DNA

Technical Field

The invention relates generally to compositions useful in "antisense" therapy and diagnosis. More particularly, it concerns compositions which are capable of binding in a sequence-specific manner to the major groove of nucleic acid duplexes and forming covalent bonds with one or both strands of the duplex.

Background Art

"Antisense" therapies are generally understood to be those which target specific nucleotide sequences associated with a disease or other undesirable condition. While the term "antisense" appears superficially to refer specifically to the well-known A-T and G-C complementarity responsible for hybridization of a "sense" strand of DNA, for example, to its "antisense" strand, this term, as applied to the technology, has come to be understood to include any mechanism for interfering with those aspects of the disease or condition which are mediated by nucleic acids. Thus, in addition to utilizing reagents which presumably hybridize by virtue of basepair complementarity to single-stranded forms such as mRNA or separated strands of DNA duplexes, materials which destroy or interfere with the function of nucleic acid duplexes are also effective.

The invention described below relates directly to this aspect of "antisense" therapy (and diagnosis). The compositions and methods useful in the invention

target the major groove of nucleic acid duplexes in sequence dependent manner. In order to distinguish targeted duplexes from those which are indigenous to the subject or which otherwise are not desired to be affected, this binding must be sequence specific.

It is now known that single-stranded oligonucleotides are capable of sequence-specific binding to the major groove in a duplex according to rules which have been reported, for example, by Moser and Dervan, Science (1987) 238:645-650. In this report, sequence-specific recognition was used to associate homopyrimidine derivatized EDTA with the major groove and effect cleavage of the double helix. Lesser degrees of sequence specificity have been designed into nonoligonucleotide molecules such as peptides, as reported by Dervan, P.B., Science (1986) 232:464-471 and by Baker and Dervan, J Am Chem Soc (1989) 111:2700-2712. The sequence-specific reagent in this pair of reports, however, resides in the minor groove of a DNA double helix.

Peptides which associate specifically with sequences in double helices are also reported by Sluka, J.P., et al., Science (1987) 238:1129-1132. Of course, peptides and proteins which regulate transcription or expression also recognize specific sequence in duplexes. In none of the foregoing reports, however, is there a covalent bond formed between the specific binding agent and the duplex.

In contrast, sequence-specific recognition of single-stranded DNA accompanied by covalent crosslinking has been reported by several groups. For example, Vlassov, V.V., et al., Nucleic Acids Res (1986) 14:4065-4076, describe covalent bonding of a single-stranded DNA fragment with alkylating derivatives of nucleotides complementary to target sequences. A report of similar work by the same group is that by Knorre, D.G., et al.,

Biochimie (1985) 67:785-789. Iverson and Dervan also showed sequence-specific cleavage of single-stranded DNA mediated by incorporation of a modified nucleotide which was capable of activating cleavage (J Am Chem Soc (1987) 109:1241-1243). Meyer, R.B., et al., J Am Chem Soc (1989) 111:8517-8519, effect covalent crosslinking to a target nucleotide using an alkylating agent complementary to the single-stranded target nucleotide sequence. A photoactivated crosslinking to single-stranded oligonucleotides mediated by psoralen was disclosed by Lee, B.L., et al., Biochemistry (1988) 27:3197-3203.

Use of N^4,N^4-ethanocytosine as an alkylating agent to crosslink to single-stranded oligonucleotides has also been described by Webb and Matteucci, J Am Chem Soc (1986) 108:2764-2765; Nucleic Acids Res (1986) 14:7661-7674. These papers also describe the synthesis of oligonucleotides containing the derivatized cytosine. Matteucci and Webb, in a later article in Tet Letters (1987) 28:2469-2472, describe the synthesis of oligomers containing N^6,N^6-ethanoadenine and the crosslinking properties of this residue in the context of an oligonucleotide binding to a single-stranded DNA.

In a recent paper, Praseuth, D., et al., Proc Natl Acad Sci (USA) (1988) 85:1349-1353, described sequence-specific binding of an octathymidylate conjugated to a photoactivatable crosslinking agent to both single-stranded and double-stranded DNA. A target 27-mer duplex containing a polyA tract showed binding of the octathymidylate in parallel along the polyA. Photoactivated crosslinking of the duplex with a p-azidophenacyl residue covalently linked to the terminus of the octathymidylate was achieved. While sequence-specific association occurred at the predicted region of the duplex, it appeared that the crosslinking reaction itself was not target specific. As photoactivation was

required to form the covalent crosslink, there could be no question of accurate sequence-specific association of the octathymidylate to the target sequence in the 27-mer duplex. A requirement for photoactivation, however, seriously limits the therapeutic potential of the crosslinking agent. Administration to a live subject does not readily admit of this mechanism of action.

In addition, Vlassov, V.V. et al., Gene (1988) 313-322 and Fedorova, O.S. et al., FEBS (1988) 228:273-276, describe targeting duplex DNA with a 5'-phospho-linked oligonucleotide.

Disclosure of the Invention

The invention provides crosslinking agents which associate in a sequence-specific manner to the major groove of nucleic acid duplexes to obtain triple helical products which are stabilized by covalent bonds. The stabilized triplex may be optionally subjected to conditions which result in cleavage of the duplex. When applied in the context of therapeutic applications, the stabilized binding of the sequence-specific crosslinking agent permits either interruption of the normal function of the duplex (for example, in replication) or, in the case of regulatable duplexes (for example, associated with transcription), may enhance the activity of the target duplex. Depending on the nature of the covalent bond formed as the crosslink, the resulting triple-helical complex may become more or less susceptible to cleavage under ambient or *in situ* conditions. Stimulation of cleavage may be desirable in the case of therapeutic regimens designed to inactivate the target DNA; it is also useful in diagnostic assays by permitting facile detection of covalently bound probes.

In one aspect, the invention is directed to crosslinking agents which associate with the major groove

of nucleic acid duplexes in a sequence-specific manner and which effect at least one covalent crosslink to at least one strand of the duplex. Multiple crosslinks may also be formed, with one or both of the duplex strands, depending on the design of the crosslinking agent. Preferred crosslinking agents are oligonucleotides, which take advantage of the duplex sequence-coupling rules known in the art, and peptide sequences, which can be designed to mimic regulatory peptides which recognize specific sequences. The moiety which performs the crosslinking function of the crosslinking agent results in the formation of covalent bonds in a pattern dependent on the design of the agent.

In an additional aspect, the invention is directed to methods to form triple helical complexes containing sequence-specific agents covalently bound in the major groove, which method comprises contacting the target duplex with a crosslinking reagent of the invention. In still other aspects, the invention is directed to the resulting triple helical complexes, and to methods for therapy and diagnosis using the crosslinking reagents of the invention.

Brief Description of the Drawings

Figure 1 shows the structures of preferred alkylating agents which effect the crosslinking of the sequence-specific agents of the invention.

Figure 2 outlines the procedure for preparation of the N^4,N^4-ethanocytosine-containing oligomers that are preferred crosslinking reagents of the invention.

Figure 3 shows the construction of a tetracassette duplex designed to assess the specificity of the reagents of the invention.

Figure 4 shows the results of an assay showing the sequence specificity of the invention crosslinking agent.

Figure 5 shows the results of treatment of target sequences with the reagents of the invention with and without cleavage of the complexes.

Modes of Carrying Out the Invention

The invention provides reagents which are capable of sequence-specific binding in the major groove of a nucleic acid duplex and which are also capable of forming covalently bonded crosslinks with the strands of the duplex without the necessity for photoactivation. As demonstrated below, moieties to effect the covalent bonding are employed which do not override the sequence specificity of the remainder of the reagent. In addition, the moiety which effects the covalently bonded crosslink is itself specific for a particular target site in a preferred embodiment.

Sequence Specificity

Sequence specificity is essential to the utility of the reagents of the invention. If not capable of distinguishing characteristic regions of a target from those of duplexes which are not to be targeted, the reagents would not behave in a manner compatible with their function as either therapeutic or diagnostic agents. Accordingly, it is essential that despite the reactivity of the moiety which effects covalent binding, this activity not be so kinetically favored that sequence specificity is lost.

Sequence specificity can be conferred in a manner consistent with the chemical nature of the reagent. In principle, the specificity is conferred by providing a region of spatial and charge distribution

which allows close association between the reagent and the charge and spatial contours of the major groove of the target duplex. This association and sequence specificity are defined in terms of the ability of the reagent to distinguish between target sequences in a sample which differ in one or more basepairs. The reagents of the invention can discriminate between regions of duplexes which differ by as few as 1 basepair out of 5, preferably 1 basepair out of 10, more preferably 1 basepair out of 15, and most preferably 1 basepair out of 20, in <u>in vivo</u> or <u>in vitro</u> culture conditions or under conditions of the diagnostic assay. The stringency of the criterion varies with the length of the region, since larger regions can tolerate more mismatches than smaller ones under the same conditions. Thus, a highly discriminatory reagent could detect a mismatch of only 1 basepair in a sequence of 20 basepairs; a more sequence-specific reagent could detect this 1-basepair difference in a region of 30 basepairs. The reagents of the invention are capable of at least discriminating between differences of 1 basepair in a 5-mer target, preferably 1 basepair in a 10-mer target, and most preferably 1 basepair in a 20-mer target.

If the sequence specificity in the reagent is conferred by an oligonucleotide, advantage can be taken of the rules for triple helix formation in the major groove, as described by Dervan (<u>supra</u>). These rules continue to be developed. For classical parallel binding of a single-stranded oligomer to a duplex, homopyrimidine stretches bind to homopurine stretches in one strand of the duplex wherein A associates with T and G with C, analogous to the complementarity rules. In this mode of association with the major groove, generally known as parallel or CT binding, the oligomer is oriented in the same direction, 5' → 3', as the homopurine stretch. An

alternate, more complex form of triple helix formation, known as GT binding, results in an antiparallel orientation.

Association of the oligonucleotide sequence specificity-conferring region of the reagent can be manipulated by utilizing either or both CT or GT binding to one or both strands of the target duplex. In co-pending application U.S. Serial No. 502,272, filed 29 March 1990, the published counterpart of which is PCT US90/06128, assigned to the same assignee and incorporated herein by reference, "switchback" oligomers are described which contain one or more regions of inverted polarity. One application of such "switchback" oligomers includes the ability to design reagents which cross over between the two strands of the duplex using parallel association with the purine regions of the strands of the duplex. Alternatively, this crossover could be effected by modifying the oligonucleotide sequence to switch back between parallel and antiparallel modes of association with the major groove. Thus, sequence specificity can be designed relative to either or both strands of the duplex.

"Oligonucleotide" is understood to include both DNA and RNA sequences and any other type of polynucleotide which is an N-glycoside or C-glycoside of a purine or pyrimidine base, or modified purine or pyrimidine base. The term "nucleoside" or "nucleotide" will similarly be generic to ribonucleosides or ribonucleotides, deoxyribonucleosides or deoxyribonucleotides, or to any other nucleoside which is an N-glycoside or C-glycoside of a purine or pyrimidine base, or modified purine or pyrimidine base. Thus, the stereochemistry of the sugar carbons may be other than that of D-ribose in certain limited residues.

"Nucleoside" and "nucleotide" include those moieties which contain not only the known purine and pyrimidine bases, but also heterocyclic bases which have been modified. Such modifications include alkylated purines or pyrimidines, acylated purines or pyrimidines, or other heterocycles. "Nucleosides" or "nucleotides" also include those which contain modification in the sugar moiety, for example, wherein one or more of the hydroxyl groups are replaced with halogen, aliphatic groups, or functionalized as ethers, amines, and the like. Examples of modified nucleosides or nucleotides include, but are not limited to:

2-aminoadenosine	2'-deoxy-2-aminoadenosine
5-bromouridine	2'-deoxy-5-bromouridine
5-chlorouridine	2'-deoxy-5-chlorouridine
5-fluorouridine	2'-deoxy-5-flurouridine
5-iodouridine	2'-deoxy-5-iodouridine
5-methyluridine	(2'-deoxy-5-methyluridine is the same as thymidine)
inosine	2'-deoxy-inosine
xanthosine	2'deoxy-xanthosine

Furthermore, as the α anomer binds to duplexes in a manner similar to that for the ß anomers, one or more nucleotides may contain this linkage.

Oligonucleotides may contain conventional internucleotide phosphodiester linkages or may contain modified forms such as phosphoramidate linkages. These alternative liking groups include, but are not limited to embodiments wherein a moiety of the formula P(O)S, $P(O)NR_2$,, P(O)R, P(O)OR', CO, or CNR_2, wherein R is H (or a salt)or alkyl (1-6C) and R' is alkyl (1-6C) is joined to adjacent nucleotides through -O- or -S-. Not all such linkages in the same oligomer need to be identical.

Inversions of polarity can also occur in "derivatives" of oligonucleotides. "Derivatives" of the oligomers include those conventionally recognized in the art. For instance, the oligonucleotides may be covalently linked to various moieties such as intercalators, substances which interact specifically with the minor groove of the DNA double helix and other arbitrarily chosen conjugates, such as labels (radioactive, fluorescent, enzyme, etc.). These additional moieties may be derivatized through any convenient linkage. For example, intercalators, such as acridine can be linked through any available -OH or -SH, e.g., at the terminal 5' position of RNA or DNA, the 2' positions of RNA, or an OH or SH engineered into the 5 position of pyrimidines, e.g., instead of the 5 methyl of cytosine, a derivatized from which contains $-CH_2CH_2CH_2OH$ or $-CH_2CH_2CH_2SH$ in the 5 position. A wide variety of substituents can be attached, including those bound through conventional linkages.

The -OH moieties in the oligomers may be replaced by phosphonate groups, protected by standard protecting groups, or activated to prepare additional linkages to other nucleotides, or may be bound to the conjugated substituent. The 5' terminal OH may be phosphorylated; the 2'-OH or OH substituents at the 3' terminus may also be phosphorylated. The hydroxyls may also be derivatized to standard protecting groups.

Methods for synthesis of oligonucleotides are found, for example, in Froehler, B. , et al., Nucleic Acids Research (1986) 14:5399-5467; Nucleic Acids Research (1988) 16:4831-4839; Nucleosides and Nucleotides (1987) 6:287-291. Froehler, B., Tet Lett (1986) 27:5575-5578; and in copending Serial No. 248,517, filed September 23, 1988, the European counterpart of which was

published based on EP application no. 89/3096347, incorporated herein by reference.

In general, there are two commonly used solid phase-based approaches to the synthesis of oligonucleotides, one involving intermediate phosphoramidites and the other involving intermediate phosphonate linkages. In both of these, the growing nucleotide chain is coupled to a solid support. In conventional methods, this linkage is as an ester formed through a succinyl residue on the support. At the termination of the synthesis, the oligonucleotide is cleaved from the solid support under nucleophilic conditions; linkage through the succinyl residue requires reasonably strong nucleophilic conditions. The standard conditions are concentrated ammonium hydroxide at 20°C for 2 hr.

Many of the oligonucleotides of the present invention which are sequence-specific binding agents to the major groove of the double helix and provide moieties capable of effecting covalent linkages, contain covalent linking moieties which are partially destroyed by these conditions. This disadvantage of solid-phase synthesis is overcome according to the present invention by utilizing an oxalyl ester linker for coupling to the solid support. This linker is cleaved under much milder conditions and the oligonucleotide can be released from the support with no significant degradation of a covalently-binding moiety such as, for example, N^4,N^4-ethanocytosine. Typical conditions for release of the oligonucleotide from the oxalyl ester are 20% aziridine in dimethylformamide for 1 hr.

With respect to the synthesis itself, in the phosphoramidite based synthesis, a suitably protected nucleotide having a cyanoethylphosphoramidite at the position to be coupled is reacted with the free hydroxyl

of a growing nucleotide chain derivatized to a solid support. The reaction yields a cyanoethylphosphonate, which linkage must be oxidized to the cyanoethylphosphate at each intermediate step, since the reduced form is unstable to acid. The phosphonate-based synthesis is conducted by the reaction of a suitable protected nucleoside containing a phosphonate moiety at a position to be coupled with a solid phase-derivatized nucleotide chain having a free hydroxyl group, in the presence of a suitable catalyst to obtain a phosphonate linkage, which is stable to acid. Thus, the oxidation to the phosphate or thiophosphate can be conducted at any point during the synthesis of the oligonucleotide or after synthesis of the oligonucleotide is complete. The phosphonates can also be converted to phosphoramidate derivatives by reaction with a primary or secondary amine in the presence of carbon tetrachloride.

Variations in the type of internucleotide linkage are achieved by, for example, using the methylphosphonates rather than the phosphonates per se, using thiol derivatives of the nucleoside moieties and generally by methods known in the art. Non-phosphorous based linkages may also be used, such as the formacetyl type linkages described and claimed in co-pending applications U.S. Serial Nos. 426,626 and 448,914, filed on 24 October 1989 and 11 December 1989, both assigned to the same assignee and both incorporated herein by reference.

In addition to employing these very convenient and now most commonly used, solid phase synthesis techniques, oligonucleotides may also be synthesized using solution phase methods such as triester synthesis. These methods are workable, but in general, less efficient for oligonucleotides of any substantial length.

The parameters which affect the ability of peptide sequences to recognize particular DNA duplex sequence targets are less well understood, but it is well known that indigenous proteins are capable of regulating transcription by selectively targeting designated regions of the duplex. In addition, as recited in the Background section above, specific peptides have been designed which are capable of the desired duplex sequence recognition. These peptides are often derivatized to additional moieties.

The sequence specificity-conferring region of the reagent is, thus, preferably an oligonucleotide and/or a peptide; i.e., combinations of these modalities may be used. However, other polymeric molecular designs which have the appropriate spatial and charge configuration to discriminate between duplex regions according to the criteria set forth above, can also be used.

Assay for Covalent Binding with Template

The ability of the candidate crosslinking reagent to effect covalent bonding to the target duplex can be assessed in simple assays using either a shift in electrophoresis gel mobility or assessment of size after cleavage. The template can be advantageously labeled at a terminus using, for example, α-P32 dATP and Klenow. The labeled template and the candidate oligonucleotide are then incubated under suitable conditions to effect triplex binding. For the shift assay they are then analyzed on a 6% denatured polyacrylamide gel after addition of an equal volume of formamide denaturant. The shift in mobility verifies binding to form the triplex and resistance to denaturation.

Reaction to form covalent linkages which then permit cleavage to be effected is demonstrated by

following the incubation to form triplex by heating with pyrolidine at 95°C for 10 min to effect the cleavage. The reaction mixture is dried down and ethanol precipitated and analyzed on 6% polyacrylamide gel.

In both of the foregoing assays, the triplex binding buffer depends on the temperature and pH of the incubation mixture. For binding at pH 6, the incubation is conducted at room temperature and the buffer contains 25 mM MOPS, 140 mM KCl, 10 mM NACl, 1 mM $MgCl_2$ and 1 mM spermine. The buffer composition is identical for pH 7.2 conditions except for the pH adjustment, and incubation is conducted at 37°C.

In the gel mobility shift assay, formation of the triplex results in a decreased mobility; when cleavage is effected, the size of the fragments is a further indication that specific covalent linkage has resulted in a cleavage-susceptible triplex.

A more sophisticated assay for sequence specificity is described below.

<u>Assay for Sequence Specificity</u>

The ability of a candidate crosslinking reagent to exhibit the required sequence specificity can readily be assessed by the procedure described in detail in the example below. Briefly, the required elements include a DNA duplex labeled at one terminus which contains individual cassettes exhibiting the level of sequence distinction desired. For example, each cassette might contain a duplex of 30 bp which differs in only one position from corresponding 30 bp structures in three other cassettes in the duplex. The candidate reagent is reacted with the labeled test DNA containing the cassettes, and the location of binding is determined. As the covalent crosslinking moiety associated with the reagent is also capable of effecting cleavage of the

duplex under appropriate conditions, the location of binding by the reagent can readily be ascertained by application of the sample to size separation techniques. Multiple binding to more than one cassette will result in multiple small fragments; binding to only one of the cassettes results in a single defined fragment of the labeled DNA of predicted size. Thus, even without prior knowledge of design rules for specific association, candidate reagents can conveniently be tested with suitably labeled cassette-containing DNA.

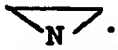
Covalent Bonding Moiety

Included in the crosslinking agent is a moiety which is capable of effecting at least one covalent bond between the crosslinking agent and the duplex. Multiple covalent bonds can also be formed by providing a multiplicity of such moieties. The covalent bond is preferably to a base residue in the target strand, but can also be made with other portions of the target, including the saccharide or phosphodiester. The reaction nature of the moiety which effects crosslinking determines the nature of the target in the duplex. Preferred crosslinking moieties include acylating and alkylating agents, and, in particular, those positioned relative to the sequence specificity-conferring portion so as to permit reaction with the target location in the strand.

If the sequence specificity-conferring portion is an oligonucleotide, the crosslinking moiety can conveniently be placed as an analogous pyrimidine or purine residue in the sequence. The placement can be at the 5' and/or 3' ends, the internal portions of the sequence, or combinations of the above. Placement at the termini to permit enhanced flexibility is preferred.

Analogous moieties can also be attached to peptide backbones.

In one particularly preferred embodiment of the crosslinking agent of the invention, a switchback oligonucleotide containing crosslinking moieties at either end can be used to bridge the strands of the duplex with at least two covalent bonds. In addition, nucleotide sequences of inverted polarity can be arranged in tandem with a multiplicity of crosslinking moieties to strengthen the complex.

Exemplary of alkylating moieties that are useful in the invention are those shown in Figure 1. These are derivatized purine and pyrimidine bases which can be included in reagents which are oligomers of nucleotides as described above. As seen in Figure 1, heterocyclic base analogs which provide alkyl moieties attached to leaving groups or as aziridenyl moieties are shown. ("Aziridenyl" refers to an ethanoamine substituent of the formula .)

It is clear that the heterocycle need not be a purine or pyrimidine; indeed the pseudo-base to which the reactive function is attached need not be a heterocycle at all. Any means of attaching the reactive group is satisfactory so long as the positioning is correct.

Additional Components of the Crosslinking Agents

While the crosslinking agents of the invention require a sequence specificity conferring portion and a moiety which effects covalent crosslinking to the duplex, the agent can also contain additional components which provide additional functions. For example, ligands which effect transport across cell membranes, specific targeting of particular cells, stabilization of the triplex by intercalation, or moieties which provide means

for detecting the oligomer alone or in the context of the triple helix formed can be included. The crosslinking agents of the invention may thus be further conjugated to lipid-soluble components, carrier particles, radioactive or fluorescent labels, specific targeting agents such as antibodies, and membrane penetrating agents and the like.

Utility and Administration

The specific crosslinking agents of the invention are useful in therapy and diagnosis. In general, in therapeutic applications, the agents are designed to target duplexes for either interruption or enhancement of their function. For example, suitable target genes for enhanced function include those which control the expression of tumor suppressor genes (Sager, Science (1989) 246:1406) or for duplexes which control the expression of cytokines such as IL-2. By redesign of the oligomer, however, complexing into the major groove may result in blocking the function of the target duplex as would be desirable where the duplex mediates the progress of a disease, such as human immunodeficiency virus, hepatitis-B, respiratory syncytial virus, herpes simplex virus, cytomegalovirus, rhinovirus and influenza virus. In addition, other undesirable duplexes are formed in various malignancies, including leukemias, lung, breast and colon cancers, and in other metabolic disorders.

The formulation of the crosslinking agents of the invention depends, of course, on their chemical nature, and on the nature of the condition being treated. Suitable formulations are available to those of ordinary skill, and can be found, for example, in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Co., Easton, PA. Dosage levels are also determined by the parameters of the particular situation, and as is

ordinarily required in therapeutic protocols, optimization of dosage levels and modes of administration are within ordinary and routine experimentation.

The crosslinking agents of the invention are particualrly useful in the treatment of latent infections such as HIV or HSV. For diagnostic use, protocols are employed which depend for their specificity on the ability of the crosslinking agent stably to bind a target double-helix region, and which permit the detection of this binding. A variety of protocols is available including those wherein the crosslinking agent is labeled to permit detection of its presence in the complex.

The following examples are intended to illustrate but not to limit the invention.

Example 1
Sequence Specific Binding of Oligomers Containing N^4N^4Ethanocytosine

Two 19-mers, Az-A:

$5'$TCTCXCTCTCTTTTTCCTT$3'$

and Az-B:

$5'$TCTCTCTCTXTTTTTCCTT$3'$

wherein X represents N^4N^4-ethanocytosine deoxynucleotide are synthesized as outlined in Figure 2. The steps in the synthesis refer to Webb and Matteucci, Nucleic Acids Res (1986) 14:5399-5467 and Froehler and Matteucci, Nucleic Acids Res (1986) 14:7661-7674; the second step is also described in Marugg et al., Tet Lett (198_) 27:2661. The 19-mers were recovered and purified using standard procedures.

Az-A and Az-B were tested for their ability to bind to a labeled diagnostic DNA containing 4 test cassettes which is diagramed in Figure 3.

As shown in Figure 3, the test cassettes contain identical sequences except for a single base. Az-A is designed to associate specifically with cassette 1; Az-B is designed to associate specifically with cassette 2. This target DNA is an end-labeled PvuII-Sal fragment containing these cassettes separated by convenient restriction sites. The N^4N^4 cytosine moiety was expected to crosslink covalently only to a guanine residue.

Four identical reactions were set up: Reaction mix 1 contained the target DNA treated with DMS which is known to effect random covalent bonding and result in multiple cleavage sites in the cassette. Reaction mix 2 contained Az-A at 50 μM; reaction mix 3 contained Az-B at 50 μM. Reaction mix 4 was another control which contained no reagent.

All reaction mixtures were a total of 10 μl and contained 1 μl 10 x buffer, which contains 1 M NaCl, 0.2 M MES, 0.1 M $MgCl_2$, pH 6.0. The target plasmid was supplied in 1 μl volume at 50,000 cpm/μl, Az-A and Az-B were supplied in 1 μl aliquots of 500 μM concentration and the volume was made up in all reaction mixtures to 10 μl with water.

The mixtures were incubated for 13.5 hr at room temperature (23-25°C).

After incubation, 1 μl DMS (1.25 dilution in H_2O) was added to reaction mix 1 and incubated for 2 min at 25°C. Then all reaction mixtures received 10 μl of 2 M freshly diluted pyrrolidine to effect cleavage at covalent binding sites and then were further incubated for 15 min at 95°C, placed on ice for 5 min and dried under vacuum.

The samples were resuspended in 25 µl water and dried under vacuum twice and then resuspended in 6 µl 67% formamide, heated for 3 min at 95°C and loaded onto a 6% denaturing polyacrylamide gel. The results of denaturing PAGE on these mixtures is shown in Figure 4.

Lane 1 represents reaction mix 1 to which DMS was added. Extensive degradation is seen. Lane 2 is the reaction mixture which contained Az-A. As shown, treatment with pyrrolidine yields mainly one degradation product, the size of which corresponds to the labeled fragment that would be obtained if cleavage occurred in cassette 1. Lane 3 shows the results from reaction mix 3 containing Az-B. Again, a single prominent degradation fragment was obtained which corresponds in size to the labeled fragment which would be obtained if cleavage occurred in cassette 2. The pyrrolidine control in lane 4 shows only modest random degradation.

As seen from a comparison of the sequences of Az-A and Az-B, each specifically recognizes the appropriate cassette differing only in one nucleotide of 19. Both also specifically covalently bind to guanine.

Example 2

Synthesis of Oligonucleotides 2-6

Several of the oligonucleotides, 2-6, as shown in Table 1, include the base analogs aziridinylcytosine (N4,N4-ethanocytosine), designated "Z" in the tabulated sequences and 5-methylcytosine, designated C' in the table. In the table, X indicates 1,3-propanediol.

Table 1

(2)	Control	5'-C'TTTTTTTC'TTTTTC'TTC'X
(3)	5'	5'-Z TTTTTTTC'TTTTTC'TTX
(4)	3'	5'-TTTTTTTC'TTTTTC'TTZX

(5)	5' + 3'	5'-Z TTTTTTTC'TTTTTC'TTZX
(6)	Internal	5'-TTTTTTTZ TTTTTC'TTX

In the oligomer synthesis, the 5-methyl-C groups were FMOC-protected and an oxalyl-CPG support (R. Letsinger, personal communication, described below) was used for the synthesis.

The synthesis scheme for aziridinylcytosine is as described in Example 1. It is incorporated into the oligomers using the standard solid phase technology modified as follows.

The base representing the 5' terminus was coupled to a CPG support for the production of the ODN_s using the following method (R. Letsinger, personal communication). Oxalyl chloride (20 μl, 0.23 mmol) was added to a solution of 1,2,4-triazole (77 mg, 1.1 mmol) in acetonitrile (2 ml). A small amount of precipitate formed but disappeared after addition of pyridine (0.1 ml). The nucleoside at the 5' terminus (0.23 mmol) in acetonitrile (1 ml) and pyridine (0.5 ml) was added, and after one hour the solution was drawn into a syringe containing aminopropylsilyl-controlled-poreglass (CPG) (400mg; 80-100 mesh, 500 A pore). This mixture was allowed to stand for 15 min. and the liquid was ejected and the solid washed four times with acetonitrile. Any residual amino groups were capped by drawing in equal volumes of THF solutions of DMAP (0.3 M) and acetic anhydride (0.6 M). The support was then washed with pyridine and acetonitrile and dried.

After the oligomers were synthesized, the support bound H-phosphonate oligomer was oxidized with I_2/pyridine/H_2O twice for 30 min and subsequently converted to the free oligonucleotide by deprotection and cleavage from the support by treatment with 20% aziridine in DMF for 2 hours at room temperature. The oligomers

were recovered and further purified by running the reaction mixture from the synthesis machine over NAP-5 (Pharmacia Sephadex G-25) column to remove salts, free aziridinylcytosine residues, FMOC blockers, etc. The NAP-5 column was used according to the manufacturers directions.

Example 3

Assay for Crosslinked Triple Helix

Oligodeoxyribonucleotides 2-6 were designed to bind the duplex target of the sequence:

5'-CCATGGA$_{10}$ $\boxed{\text{GAAAAAAAGAAAAAGAAG}}$ AAATTTCTTTTTCTTTCT$_{12}$...p*

As a comparison of the squared portion of the duplex to the sequences in Figure 1 will demonstrate, the potentially covalent binding moiety, Z, is at the 3' terminus of the oligomer in ODN3, at the 3' end in ODN4, at both ends in ODN5 and internal to the oligomer in ODN6.

Each of these oligomers were incubated with the duplex using the triplex binding buffer as set forth above at pH 7.2 at 37°C for 2 hr. The reactions were quenched with pyrolidine, heated and evaporated as described above before subjecting the mixtures to denaturing PAGE. The treatment results in cleavage of the duplex at the site of covalent bonding as described by Maxam, A. et al., Proc Natl Acad Sci USA (1977) 74:560.

The results are shown in Figure 5. In Figure 5, lane 1 represents the untreated duplex target, and shows no difference from lane 2 which was treated with ODN2, containing no crosslinking moiety. Lanes 3 and 4 represent the results of reaction mixtures using ODNs 3 and 4 respectively; in both cases, considerable reaction

has occurred; this reaction is virtually complete in lane 5 which represents treatment with ODN5. Lane 6 indicates that although some reaction occurred with ODN6, this was less effective when the covalent binding moiety is internal to the oligomer.

Lanes 7-10 represent the alternate form of the assay described hereinabove wherein a mobility shift is detected, rather than cleavage. In the samples applied to these lanes, the reaction was stopped not with pyrolidine but with the denaturing agent formamide. Lane 7 represents the target duplex only, lane 8 the target with ODN2 containing no covalently-binding moiety, and lanes 9 and 10 contain reaction mixtures of the duplex with ODNs 3 and 4 respectively. As shown in Figure 5, the lower mobility is reflected in cases where the covalent bonding is effected. Denaturation with the formamide destroys the triplex when no crosslinking moiety is present.

In addition, the foregoing techniques were used to assess the kinetics of the crosslinking reaction. The half-life of the reaction was approximately 1 hr for ODN4 with the concentration of ODN4 at 1 μM; ODN3 which has the analog at the 5' position showed a rate approximately four times slower. ODN4 provided virtually 100% crosslinking after 16 hr.

Example 4

Additiional Crosslinking Agents

In the illustrative oligonucleotides set forth below, the following notation is used: The modified nucleoside N-methyl-8-oxo-2'-deoxyadenine (MODA) is designated "M"; 5-methylcytosine is represented by "Ć"; and nucleosides containing an aziridenyl group (N^4N^4-ethanocytosine) are designated "Z".

In addition, some of the oligomers contain an inverted polarity region, in this illustration formed from an o-xyloso dimer synthon. The linking group, o-xyloso (nucleotides that have xylose sugar linked via the o-xylene ring), is designated "X".

Crosslinking agents that bind to certain HIV targets are as follows. For binding to the 5'-GGAAAAGGAAGGAAATTTC-3' sequence:

111 5'-MMTTTTMMTTMMT-X^1-TTM-5';
112 5'-MMTTTTMMTTMMT-X^1-TTZ-5';
113 5'-ZMTTTTMMTTMMT-X^1-TTZ-5';
114 5'-ZMTTTTMMTTMMT-X^1-TTM-5';
115 5'-MĆTTTTMĆTTMĆT-X^1-TTM-5';
116 5'-MĆTTTTMĆTTMĆT-X^1-TTZ-5';
117 5'-ZĆTTTTMĆTTMĆT-X^1-TTZ-5'; and
118 5'-ZĆTTTTMĆTTMĆT-X^1-TTM-5'.

For binding to the 5'-AGAGAGAAAAAAGAG-3' sequence:

131 5'-TĆTĆTĆTTTTTTĆTĆ-3';
132 5'-TĆTĆTĆTTTTTTĆTZ-3';
133 5'-ZTĆTĆTTTTTTĆTZ-3'; and
134 5'-MTMTMTTTTTTMTZ-3'.

For binding to the 5'-AAGAGGAGGAGGAGG-3' sequence:

141 5'-TTĆTMĆTMĆTMĆTMZ-3';
142 5'-TTĆTMMTMMTMMTMZ-3'; and
143 5'-TTĆTĆMTĆMTĆMTĆZ-3'.

For binding to the 5'-AGAAGAGAAGGCTTTC-3' sequence:

152 5'-TĆTTĆTĆTTM-X^2-TTZ-5'; and
156 5'-TMTTMTMTTM-X^2-TTZ-5'.

The oligonucleotides are labeled by kinasing at the 5' end and are tested for their ability to bind target sequence under conditions of 1 mM spermine, 1 mM $MgCl_2$, 140 mM KCl, 10 mM NaCl, 20 mM MOPS, pH 7.2 with a

target duplex concentration of 10 pM at 37°C for 1 hour. These conditions approximate physiological conditions, and the binding is tested either in a footprint assay, or in a gel-shift assay essentially as described in Cooney, M. et al., Science (1988) 241:456-459.

For oligomers designed to target Human Interleukin-1 Beta Gene (HUMIL1B), illustrative nucleotides are :

a. for HUMIL1B beginning at neucleotide 6379

104 5'-ZTTTTMTTMTM-X^1-TMTTTT-5',

b. for HUMIL1B beginning at neucleotide 7378

112 5'-ZTTĆTTTTTTTTT-X^2-ĆTTTĆMT-5',
114 5'-MTTMTTTTTTTTT-X^2-MTTTMZ-5',
115 5'-ZTTMTTTTTTTTT-X^2-MTTTMZ-5',
116 5'-ZTTMTTTTTTTTT-X^2-MTTTMM-5'.

For oligomers designed to target Human Tumor Necrosis Factor (HUMTNFAA), the illustrative nucleotides are:

a. for HUMTNFAA beginning at neucleotide 251

203 5'-TMTMMMTTM-X^3-MMMMZ-5',

b. for HUMTNFAA beginning at neucleotide 1137

212 5'-ZMMMTTĆTĆTĆTĆTĆTĆTTTĆT-3',
214 5'-MMMMTTĆTĆTĆTĆTĆTĆTTTZ-3',
215 5'-ZMMMTTĆTĆTĆTĆTĆTĆTTTZ-3',
216 5'-ZMMMTTĆTĆTĆTĆTĆTĆTTTM-3',
218 5'-MMMMTTMTMTMTMTMTMTTTZ-3',
219 5'-ZMMMTTMTMTMTMTMTMTTTZ-3',
220 5'-ZMMMTTMTMTMTMTMTMTTTM-3'.

For oligomers designed to target Human Leukocyte Adhesion Protein p150,95 Alpha Subunit Gene (HUMINT02), illustrative nucleotides are:

a. for HUMINT02 beginning at neucleotide 1612

302 5'-TĆTTMĆTT-X^4-MTTĆTMZ-5',
304 5'-TMTTMMTT-X^4-MTTMTMZ-5',

For oligomers designed to target Human Interleukin-2 Receptor Gene (HUMIL2R8), the exon 8 target and flanks, illustrative nucleotides are:

a. for HUMIL2R8 beginning at neucleotide 1114
502 5'-TTMĆTTMĆTTTĆTTTĆTTMĆTTZ-3',
504 5'-MMTTMMTTTMTTTMTTMMTTZ-3',
505 5'-ZMTTMMTTTMTTTMTTMMTTM-3',
506 5'-ZMTTMMTTTMTTTMTTMMTTZ-3',

b. for HUMIL2R8 beginning at neucleotide 1136
512 5'-ZTTĆTMMMTĆTTMMMT-3'.

For oligomers designed to target Human Interleukin-4 Gene (HUMIL4), the illustrative nucleotides are:

a. for HUMIL4 beginning at neucleotide 75
602 5'-TMTMMMMMTTZ-3',

b. for HUMIL4 beginning at neucleotide 246
612 5'-ZTĆTTMMT-X^{6}-MTTMT-3',
614 5'-ZTMTTMMT-X^{6}-MTTMT-3'.

For oligomers designed to target Human Interleukin-6 Receptor Gene (HUMIL6), the illustrative nucleotides are:

a. for HUMIL6 beginning at neucleotide 2389
702 5'-ZMMMTTĆT-X^{6}-TMTMTMMTMMMTTTMTTMMT-5',
704 5'-MMMMTTĆT-X^{6}-TĆTĆTĆĆTMMMTTTMTTMMZ-5',
705 5'-ZMMMTTĆT-X^{6}-TĆTĆTĆĆTMMMTTTMTTMMZ-5',
706 5'-ZMMMTTĆT-X^{6}-TĆTĆTĆĆTMMMTTTMTTMMM-5',

b. for HUMIL6 beginning at neucleotide 2598
712 5'-TMTMMTTMMTMTMMTMTMMMZ-3',
714 5'-TMTMĆTTMĆTMTMĆTMTMMMZ-3'.

For oligomers designed to target Human Interleukin-6 Gene (HUMIL6B), the sequence beginning at neucleotide 18, the illustrative nucleotides are:

802 5'-ZTMMMMTTMTM-X^{1}-TTMT-5'.

For oligomers designed to target Human Interferon-Gamma Gene (HUMINTGA), the sequence beginning at neucleotide 295, the illustrative nucleotides are:

812 5'-MMTTTMTMMTMTZ-3',
813 5'-ZMTTTMTMMTMTZ-3',
814 5'-ZMTTTMTMMTMTM-3'.

For oligomers designed to target Human Interleukin-1 Receptor Gene (HUMIL1RA), the illustrative nucleotides are:

a. for HUMIL1RA beginning at neucleotide 3114
912 5'-TTTMMTMMTMMTTMMZ-3',
914 5'-TTTMĆTMĆTMĆTTMMZ-3'.

For oligomers designed to target Human Tumor Necrosis Factor Receptor mRNA (HUMNFR), the sequence beginning at nucleotide 2354:

942 5'-TTTTĆTTTTTTTTTTTTZ-3',
943 5'-TTTTMTTTTTTTTTTTTZ-3'.

For oligomers designed to target Human Hepatitis B Virus (HBV), the illustrative nucleotides are:

a. for HBV beginning at nucleotide 2365
101 5'-TĆTTĆTTĆT-X^{1}-MMMTM-5',
102 5'-TĆTTĆTTĆT-X^{1}-MMMTZ-5',
103 5'-TMTTMTTMT-X^{1}-MMMTM-5',
104 5'-TMTTMTTMT-X^{1}-MMMTZ-5',

b. for HBV beginning at nucleotide 2605
111 5'-MTĆTTTTĆTTĆT-3',
112 5'-ZTĆTTTTĆTTĆT-3',
113 5'-MTMTTTTMTTMT-3',
114 5'-ZTMTTTTMTTMT-3'.

For oligomers designed to target Human Papilloma Virus Type 11 (HPV-11), the illustrative nucleotides are:

a. for HPV-11 beginning at nucleotide 927
201 5'-MTMĆTTĆTMĆTMĆ-3',

202 5'-ZTMĆTTĆTMĆTMĆ-3',

b. for HPV-11 beginning at nucleotide 7101

211 5'-TTTTĆTTT-X^1-TTTM-5',

212 5'-TTTTĆTTT-X^1-TTTZ-5',

213 5'-TTTTMTTT-X^1-TTTM-5',

214 5'-TTTTMTTT-X^1-TTTZ-5'.

For oligomers designed to target Human Papilloma Virus Type 16 (HPV-16), the sequence beginning at nucleotide 6979, the illustrative nucleotides are:

301 5'-TTTMĆTTT-X^1-TTĆT-5',

302 5'-TTTMMTTT-X^1-TTMT-5'.

For oligomers designed to target Human Respiratory Syncytial Virus (RSV), the illustrative nucleotides are:

a. for RSV beginning at nucleotide 1307

401 5'-TMĆTTĆTĆTTĆT-3',

402 5'-TMMTTMTMTTMT-3',

403 5'-TĆĆTTMTMTTMT-3',

b. for RSV beginning at nucleotide 5994

411 5'-TTĆTTTTMĆTTTTĆT-X^1-TTĆTT-5',

412 5'-TTMTTTTMMTTTTMT-X^1-TTMTT-5'.

For oligomers designed to target Herpes Simplex Virus II (HSV II IE3), the illustrative nucleotides are:

501 5'-MTĆTTĆTTĆTT-X^2-MĆMĆMĆMĆM-5',

502 5'-MTĆTTĆTTĆTT-X^2-MĆMĆMĆMĆZ-5',

503 5'-ZTĆTTĆTTĆTT-X^2-MĆMĆMĆMĆZ-5',

504 5'-ZTĆTTĆTTĆTT-X^2-MĆMĆMĆMĆM-5',

505 5'-MTĆTTĆTTĆTT-X^2-MMMMMMMMM-5',

506 5'-MTĆTTĆTTĆTT-X^2-MMMMMMMMZ-5',

507 5'-ZTĆTTĆTTĆTT-X^2-MMMMMMMMZ-5',

508 5'-ZTĆTTĆTTĆTT-X^2-MMMMMMMMM-5',

509 5'-MTMTTMTTMTT-X^2-MMMMMMMMM-5',

510 5'-MTMTTMTTMTT-X^2-MMMMMMMMZ-5',

511 5'-ZTMTTMTTMTT-X^2-MMMMMMMMZ-5',

512 5'-ZTMTTMTTMTT-X^2-MMMMMMMMM-5'.

For oligomers designed to target Herpes Simplex Virus II (HSV II Ribonucleotide Reductase), the illustrative nucleotides are:

601 5'-MTMMMMMM-X^3-ĆTTĆTTM-5',
602 5'-MTMMMMMM-X^3-ĆTTĆTTZ-5',
603 5'-ZTMMMMMM-X^3-ĆTTĆTTZ-5',
604 5'-ZTMMMMMM-X^3-ĆTTĆTTM-5',
605 5'-MTMMMMMĆ-X^3-MTTMTTM-5',
606 5'-MTMMMMMĆ-X^3-MTTMTTZ-5',
607 5'-ZTMMMMMĆ-X^3-MTTMTTZ-5',
608 5'-ZTMMMMMĆ-X^3-MTTMTTM-5'.

For oligomers designed to target Herpes Simplex Virus I (HSV), the illustrative nucleotides are:

a. for HSV beginning at nucleotide 52916

701 5'-MMMTTTMĆTTTMTMĆTTT-3',
702 5'-MMMTTTMMTTTMTMMTTT-3',
703 5'-MMMTTTĆĆTTTMTĆĆTTT-3',

b. for HSV beginning at nucleotide 121377

711 5'-MTMMMTM-X^3-TMĆTĆTT-5',
712 5'-ZTMMMTM-X^3-TMĆTĆTT-5',
713 5'-MTMMMTM-X^3-TMMTMTT-5',
714 5'-ZTMMMTM-X^3-TMMTMTT-5',

c. for HSV beginning at nucleotide 10996

721 5'-MMMMMTĆTMMM-X^1-TMMMTĆT-5',
722 5'-ZMMMMTĆTMMM-X^1-TMMMTĆT-5',
723 5'-MMMMMTMTMMM-X^1-TMMMTMT-5',
724 5'-ZMMMMTMTMMM-X^1-TMMMTMT-5'.

For oligomers designed to target Cytomegalovirus (CMV), the illustrative nucleotides are:

a. for CMV beginning at nucleotide 176

801 5'-MMMMTTTTMTMMT-X^1-TMMM-5',
802 5'-MMMMTTTTMTMĆT-X^1-TMMM-5',
803 5'-MMMMTTTTMTMĆT-X^1-TMMZ-5',
804 5'-ZMMMTTTTMTMĆT-X^1-TMMZ-5',
805 5'-ZMMMTTTTMTMĆT-X^1-TMMM-5',

b. for CMV beginning at nucleotide 37793

811 5'-MMMTTĆTM-X^3-ĆTTĆTMMMM-5',
812 5'-MMMTTĆTM-X^3-ĆTTĆTMMMZ-5',
813 5'-ZMMTTĆTM-X^3-ĆTTĆTMMMZ-5',
814 5'-ZMMTTĆTM-X^3-ĆTTĆTMMMM-5',
815 5'-MMĆTTMTM-X^3-MTTMTMMMM-5',
816 5'-MMĆTTMTM-X^3-MTTMTMMMZ-5',
817 5'-ZMĆTTMTM-X^3-MTTMTMMMZ-5',
818 5'-ZMĆTTMTM-X^3-MTTMTMMMM-5',

c. for CMV beginning at nucleotide 7304

821 5'-MMMMTMĆTĆTMĆTĆTĆTĆTTĆTMĆTM-3',
822 5'-MMMMTMĆTĆTMĆTĆTĆTĆTTĆTMĆTZ-3',
823 5'-MMMMTMMTMTMMTMTMTMTTMTMMTM-3',
824 5'-MMMMTMMTMTMMTMTMTMTTMTMMTZ-3',
825 5'-ZMMMTMMTMTMMTMTMTMTTMTMMTZ-3',
826 5'-ZMMMTMMTMTMMTMTMTMTTMTMMTM-3',
827 5'-MMMMTĆĆTMTĆĆTMTMTMTTMTĆĆTM-3',
828 5'-MMMMTĆĆTMTĆĆTMTMTMTTMTĆĆTZ-3',
829 5'-ZMMMTĆĆTMTĆĆTMTMTMTTMTĆĆTZ-3',
830 5'-ZMMMTĆĆTMTĆĆTMTMTMTTMTĆĆTM-3'.

Claims

1. A peptide or oligonucleotide crosslinking agent that binds in the major groove of a nucleic acid duplex in a sequence-specific manner, and which agent forms, without photoactivation, a covalent crosslink at at least one site of said duplex, said agent comprising a region conforming sequence-specificity and a moiety which effects a covalent crosslink through a residue of the peptide or a base of the oligonucleotide.

2. The crosslinking agent of claim 1 wherein the sequence specificity conferring region is an oligonucleotide or derivative thereof.

3. The crosslinking agent of claim 1 which comprises a multiplicity of moieties which effect crosslinks to the duplex.

4. The crosslinking agent of claim 1 wherein said moiety which effects crosslinking is an alkylating agent.

5. The crosslinking agent of claim 4 wherein said alkylating agent is an ethanoamino moiety.

6. The crosslinking agent of claim 5 wherein said alkylating agent is an N,N-ethanopurine or N,N-ethanopyrimidine.

7. The crosslinking agent of claim 1 wherein the moiety which effects crosslinking is a substituent of the agent selected from the group consisting of formulas 1-4 of Figure 1.

8. The crosslinking agent of claim 1 wherein said sequence specificity region distinguishes regions of the target duplex which differ by 1 bp in a sequence of 5 bp.

9. A triple helical complex which comprises a nucleic acid duplex containing the crosslinking agent of claim 1 in its major groove.

10. A method to form a covalently bonded triple helical complex with a sequence-specific agent crosslinked in the major groove, which method comprises contacting a nucleic acid duplex with the crosslinking agent of claim 1 under conditions which favor formation of said complex.

11. A method to control diseases or conditions in an animal subject, which diseases or conditions are mediated by nucleic acid duplex, which method comprises administering to a subject in need of such treatment an effective amount of the crosslinking agent of claim 1.

12. The method of claim 11 wherein said disease or condition is a latent infection.

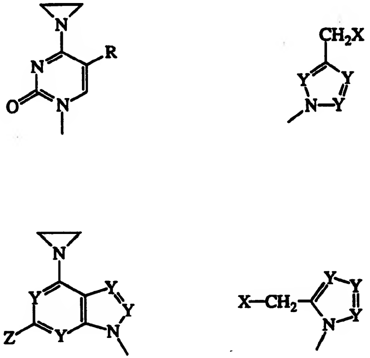
13. A method to detect a nucleic acid duplex containing a target sequence of nucleotides, which method comprises:

contacting a sample suspected to contain said duplex with a crosslinking agent capable of covalently binding to the major groove of the duplex in a manner specific to said target sequence under conditions wherein said duplex and crosslinking reagent form a complex, and

detecting the formation of at least one crosslink in said complex.

14. The method of claim 13 wherein said detecting comprises treating said complex with a denaturing agent and subjecting the resultant to denaturing electrophoresis, and wherein complexes containing said crosslink exhibit lowered mobility.

15. A method to synthesize an oligonucleotide containing at least one nucleotide residue having an ethanoamino moiety as a substituent on the base portion thereof which method comprises conducting solid-phase synthesis of said oligomer in a solid-phase system wherein the oligomer intermediates are coupled to the solid phase through an oxalyl moiety.



R = H, alkyl
U = O, S
X = Leaving Group
Y = N,CH
Z = H, NH_2, NHR

Figure 1

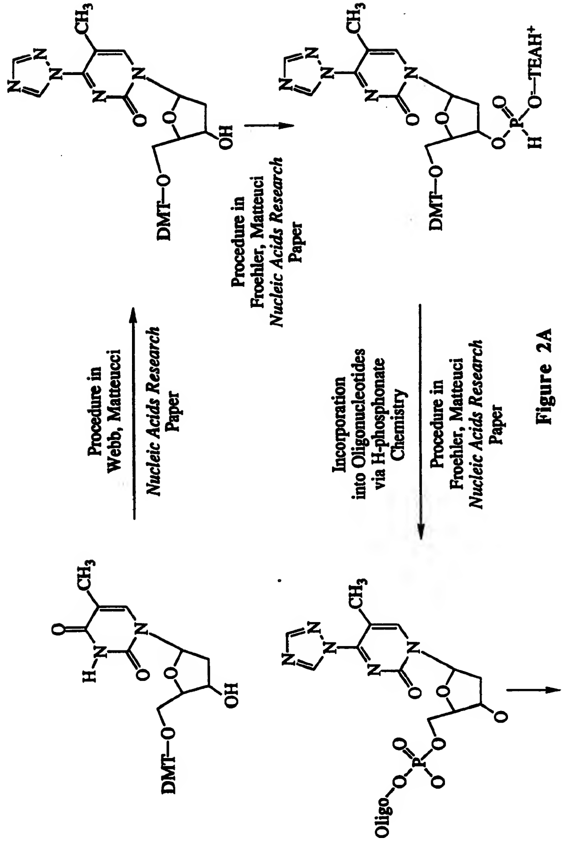
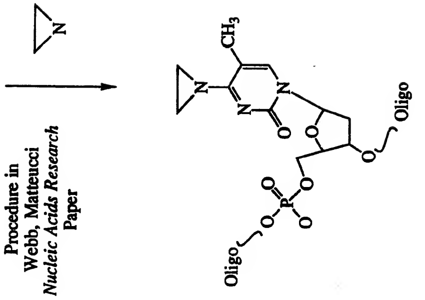

Procedure in
Webb, Matteucci
Nucleic Acids Research
Paper
Procedure in
Froehler, Matteuci
Nucleic Acids Research
Paper
Incorporation
into Oligonucleotides
via H-phosphonate
Chemistry
Procedure in
Froehler, Matteuci
Nucleic Acids Research
Paper

Figure 2A


Procedure in
Webb, Matteucci
Nucleic Acids Research
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CH3
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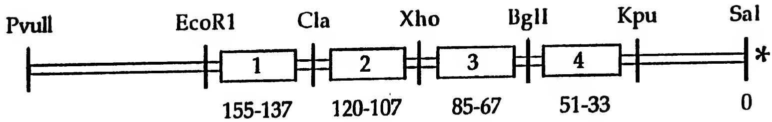
Oligo

Figure 2B

Triplex Cassette Fragment #1

(Single Base Discrimination)




```
Oligonucleotide:             (5') T C T C X C T C T C T T T T T C C T (3')
                                  • + • + I + • + • + • • • • • + + •
Purine Strand
Target:   Cassette 1         (5') A G A G G G A G A G A A A A A G G A A G A G A A G (3')
          Cassette 2                   A
          Cassette 3                   T
          Cassette 4                   C
```

Fig. 3

FIG. 4

International Application No PCT/US91/03680

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12P 19/34; C12Q 1/00, 1/64; G01N 33/00, 33/564, 33/566

US Cl: 435/4, 6, 91; 436/63, 94, 501, 508

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S. Cl:	435/4, 6, 91 436/63, 94, 501, 508

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Nucleic Acids Research, volume 14, Number 19, issued 1986, T.R. Webb et al., "Hybridization Triggered Cross-Linking of Deoxyoligonucleotides," pages 7661-7674, see entire document.	1-8,11,12, and 15
Y	Proceedings of the National Academy of Science, volume 85, issued March 1988, D. Praseuth et al., "Sequence-specific Binding and Photocrosslinking of ≤ and B oligonucleotide to the major Groove of DNA via Triple-Helix Formation," pages 1349-1353, see entire document.	1-8,11,12, and 15

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 August 1991	12 SEP 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Jeanne Mosie for Lori Yuan

Form PCT/ISA/210 (second sheet) (Rev.11-87)

International Application No. PCT/US91/03680

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers__________, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See attached sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

   1-8,11,12,15

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Attachment to PCT 210

I. Claims 1-8, 11, 12, and 15 drawn to a first product of a crosslinking agent, a first method of using first product, and a first method of making first product, Class 536, subclass 27 and Class 435, subclass 87.

II. Claim 9 drawn to a second product of a triple helical complex, Class 536, subclass 27.

III. Claim 10 drawn to a method of making second product, Class 435, subclass 91.

IV. Claims 13 and 14 drawn to a second use of first product, Class 435, subclass 6.